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# Processed cheese products — Determination of nitrogen content and crude protein calculation — Kjeldahl method

*Fromages fondus — Détermination de la teneur en azote et calcul des protéines brutes — Méthode Kjeldahl*

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# Foreword

**ISO (the International Organization for Standardization)** is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 17837 | IDF/RM 25 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

This corrected version of ISO/TS 17837:2008 | IDF/RM 25 incorporates the following corrections:

a) the introductory element of the title on the cover and page 1, “Milk and milk products”, has been modified to “Processed cheese products”, the corresponding change in the French title on the cover being the deletion of “*Lait et produits laitiers*” and insertion of “*Fromages fondus*”;

b) in the ISO foreword, the penultimate paragraph (a previous edit of the final paragraph) has been deleted;

c) Clause 1 and its Note have been modified to relate solely to processed cheese products;

d) the title “8.1 Cheese” and the whole of 8.2 have been deleted, with corresponding updates to the contents list;

e) in 9.1.1, line 2, and in 9.2.1, line 2, “8.1 or 8.2” has been deleted, and “Clause 8” inserted;

f) in 9.1.2.1, paragraph 2, line 2, and 9.2.2.1, paragraph 5, line 2, “for milk analysis” has been deleted.

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# Foreword

**IDF (the International Dairy Federation)** is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented at the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a Standing Committee may decide to publish another type of normative document which is called by IDF: *Reviewed method*. Such a method represents an agreement between the members of a Standing Committee and is accepted for publication if it is approved by at least 50 % of the committee members casting a vote. A *Reviewed method* is equal to an ISO/PAS or ISO/TS and will, therefore, also be published jointly under ISO conditions.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

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All work was carried out by the Joint ISO-IDF Action Team on *Nitrogen compounds* of the Standing committee on *Main components in milk* under the aegis of its project leader, Mr. J. Romero (US).

This edition of ISO/TS 17837 | IDF/RM 25 cancels and replaces IDF 25:1964, which has undergone minor editorial and technical revisions.

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# Processed cheese products — Determination of nitrogen content and crude protein calculation — Kjeldahl method

**WARNING — Performance of the method specified in this Technical Specification may involve the use of hazardous materials, operations, and equipment. This Technical Specification does not purport to address all the safety risks associated with such performance. It is the responsibility of the user to establish appropriate safety and health practices and determine the applicability of local regulatory limitations prior to performance of the method.**

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## 1 Scope

This Technical Specification specifies a method for the determination of the nitrogen content and crude protein content by calculation in processed cheese products by using the Kjeldahl principle, both traditional and block digestion methods.

NOTE Inaccurate crude protein results are obtained if non-dairy sources of nitrogen are present in the specified processed cheese products.

## 2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 385, *Laboratory glassware — Burettes*

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

ISO 4788, *Laboratory glassware — Graduated measuring cylinders*

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

**3.1**
**nitrogen content**
mass fraction of nitrogen determined by the procedure specified in this Technical Specification

NOTE The nitrogen mass fraction is expressed as a percentage.

**3.2**
**crude protein content**
mass fraction of crude protein calculated as specified by this Technical Specification

NOTE The crude protein mass fraction is expressed as a percentage.

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## 4 Principle

A test portion is digested with a mixture of concentrated sulfuric acid and potassium sulfate. Copper(II) sulfate is used as a catalyst to thereby convert organic nitrogen present to ammonium sulfate. The function of the potassium sulfate is to elevate the boiling point of the sulfuric acid and to provide a stronger oxidizing mixture for digestion. Excess sodium hydroxide is added to the cooled digest to liberate ammonia. The liberated ammonia is steam distilled into excess boric acid solution and titrated against a hydrochloric acid standard volumetric solution. The nitrogen content is calculated from the amount of ammonia produced and the crude protein content from the nitrogen content obtained.

## 5 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade, and only distilled or demineralized water or water of equivalent purity.

**5.1 Potassium sulfate** ($K_2SO_4$), nitrogen free.

**5.2 Copper(II) sulfate pentahydrate solution**, $\rho(CuSO_4 \cdot 5H_2O)$ = 5,0 g/100 ml.

Dissolve 5,0 g of copper(II) sulfate pentahydrate in water in a 100 ml one-mark volumetric flask (6.8). Dilute to the mark with water and mix.

**5.3 Sulfuric acid** ($H_2SO_4$), with a mass fraction of between 95 % and 98 %, nitrogen free [$\rho_{20}(H_2SO_4) \approx 1{,}84$ g/ml].

**5.4 Sodium hydroxide aqueous solution**, nitrogen free, containing 50 g of sodium hydroxide (NaOH) per 100 g (mass fraction of sodium hydroxide, $w_{NaOH}$ = 50 %).

If plugging of the flow system in an automatic distillation unit is a problem, use a solution with $w_{NaOH}$ = 40 %.

**5.5 Indicator solution**.

**5.5.1** Dissolve 0,1 g of methyl red in 95 % (volume fraction) ethanol in a 50 ml one-mark volumetric flask (6.8). Dilute to the 50 ml mark with similar ethanol and mix.

**5.5.2** Dissolve 0,5 g of bromocresol green in 95 % (volume fraction) ethanol in a 250 ml one-mark volumetric flask (6.8). Dilute to the mark with similar ethanol and mix.

**5.5.3** Mix one volume of the methyl red solution (5.5.1) with five volumes of the bromocresol green solution (5.5.2) or combine and mix all of both solutions.

**5.6 Boric acid solution**, $\rho(H_3BO_3)$ = 40,0 g/l.

Dissolve 40,0 g of boric acid ($H_3BO_3$) in 1 l hot water in a 1 000 ml one-mark volumetric flask (6.8). Allow the flask and its contents to cool to 20 °C. Make up to the mark with water, add 3 ml of indicator solution (5.5.3) and mix.

Store the solution, which is light orange in colour, in a borosilicate glass bottle. Protect the solution from light and sources of ammonia during storage.

NOTE If using electronic pH end-point titration, the addition of the indicator solution (5.5.3) to the boric acid solution can be omitted. On the other hand, the change in colour can also be used as a check on proper titration procedures.

**5.7 Hydrochloric acid standard solution**, $c$(HCl) = (0,1 ± 0,000 5) mol/l.

The purchase of pre-standardized hydrochloric acid standard solution from a reputable manufacturer is recommended.

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Using pre-standardized solutions avoids introduction of systematic errors when diluting a concentrated stock hydrochloric acid solution and then determining the molarity of the acid, a process which can give rise to poor reproducibility performance of the method. It also avoids the use of a standard solution for titration having a higher concentration than the mentioned upper limit (0,1 ± 0,000 5 mol/l), as that reduces the total titration volume per sample. In the latter case, the uncertainty in readability of the burette becomes a larger percentage of the value, which has a negative impact on the repeatability and reproducibility performance of the method. The same issues and additional sources of error arise when another acid (e.g. sulfuric acid) is substituted for hydrochloric acid. Such substitutions are therefore not recommended.

**5.8 Ammonium sulfate** [$(NH_4)_2SO_4$], with a minimum mass fraction assay of 99,9 % on dried material.

Immediately before use, dry the ammonium sulfate at 102 °C ± 2 °C for 2 h. Cool to room temperature in a desiccator.

**5.9 Tryptophan** ($C_{11}H_{12}N_2O_2$) or **lysine hydrochloride** ($C_6H_{14}N_2O_2$·HCl), with a minimum mass fraction assay of 99 %. When stored in a desiccator, it is not necessary to dry these reagents in an oven before use.

**5.10 Sucrose** ($C_{12}H_{22}O_{11}$), with a mass fraction of nitrogen of less than 0,002 %. Do not dry the sucrose in an oven before use.

## 6 Apparatus

Usual laboratory apparatus and, in particular, the following.

**6.1 Kjeldahl flasks**, of capacity 500 ml or 800 ml.

**6.2 Analytical balance**, capable of weighing to the nearest 0,1 mg.

**6.3 Burette or automatic pipette**, capable of delivering portions of 1,0 ml of copper(II) sulfate solution (5.2).

**6.4 Graduated measuring cylinders**, of capacities 50 ml, 100 ml and 500 ml, complying with the requirements of ISO 4788, class A.

**6.5 Conical flasks**, of capacity 500 ml, graduated at every 200 ml.

**6.6 Burette**, of capacity 50 ml, graduated at least at every 0,1 ml, complying with the requirements of ISO 385, class A. Alternatively, an automatic burette can be used fulfilling the same requirements.

**6.7 Grinding device**.

**6.8 One-mark volumetric flasks**, of capacities 50 ml, 100 ml, 250 ml, and 1 000 ml, complying with the requirements of ISO 1042, class A.

**6.9 Boiling aids**, e.g. calcined pumice, zinc dust, hard pieces of porcelain or high-purity amphoteric alundum (i.e. carborundum) granules, plain, mesh size 10. Do not reuse the aids.

NOTE Glass beads of approximately 5 mm diameter can also be used, but they may not promote boiling as efficiently as the alundum granules. More foaming problems may be encountered during digestion with glass beads.

**6.10 Digestion apparatus**, to hold the Kjeldahl flasks (6.1) in an inclined position (approximately 45°), with electric heaters or gas burners that do not heat the flasks above the level of their contents, and with a fume extraction system.

The heater source should be adjustable to control the maximum heater setting to be used during digestion. Preheat the heat source at the heater setting for evaluation. In the case of a gas heater, the preheated period shall be 10 min and for an electric heater, it shall be 30 min. For each of the heaters, determine the heater

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setting that brings 250 ml of water including 5 to 10 boiling aids with an initial temperature of 25 °C to its boiling point in 5 min to 6 min. This is the maximum heater setting to be used during digestion.

**6.11 Distillation apparatus** (traditional method), made of borosilicate glass or other suitable material to which can be fitted a Kjeldahl flask (6.1) consisting of an efficient splash-head connected to an efficient condenser with straight inner tube and an outlet tube attached to its lower end. The connecting tubing and stopper(s) shall be close-fitting and preferably made of neoprene.

NOTE The distillation apparatus mentioned above may be replaced by the complete Parnas-Wagner distillation configuration (see Reference [4]) or other suitable equipment.

**6.12 Digestion block**, aluminium alloy block or equivalent block, fitted with an adjustable temperature control and device for measuring block temperature.

**6.13 Digestion tubes**, of capacity 250 ml, suitable for use with the digestion block (6.12).

**6.14 Exhaust manifold**, suitable for use with the digestion tubes (6.13).

**6.15 Centrifugal scrubber apparatus** or **filter pump** or **aspirator**, constructed of acid resistant material, for use with mains water supply.

**6.16 Distillation unit** (block digesting method), capable of steam distilling, manual or semi-automatic, suitable for accepting digestion tubes (6.13) and conical flasks (6.5).

**6.17 Automatic titrator**, provided with a pH-meter.

The pH-meter shall be calibrated properly in the range of pH 4 to pH 7 following normal laboratory pH-calibration procedures.

**6.18 Spatula** or suitable transfer device.

**6.19 Filter paper**, nitrogen-free, of dimensions and porosity suitable to hold the cheese test portion.

**6.20 Water bath**, capable of maintaining a temperature of between 38 °C and 40 °C.

## 7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50[1].

## 8 Preparation of the test sample

Remove the rind, smear or mouldy surface layer of the cheese, in such a way as to provide a test sample representative of the cheese as it is usually consumed.

Grind (6.7) the representative test sample thus obtained. Quickly mix the whole mass and preferably grind the mass again quickly. Analyse the test sample as soon as possible after grinding.

Using a spatula (6.18), weigh 1 g of ground cheese on to a pre-folded, tarred filter paper (6.19). Enclose the test sample in filter paper and drop the whole on to the bottom of a Kjeldahl flask (6.1) or digestion tube (6.13) as indicated in 9.1.1 or 9.2.1.

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# 9 Procedure

## 9.1 Traditional method

### 9.1.1 Test portion and pre-treatment

Add to a clean and dry Kjeldahl flask (6.1), 5 to 10 boiling aids (6.9), 15,0 g of the potassium sulfate (5.1), 1,0 ml of the copper(II) sulfate solution (5.2). Then add the prepared test sample as indicated in Clause 8, and 25 ml of the sulfuric acid (5.3) while using the sulfuric acid to wash down any copper(II) sulfate solution, potassium sulfate or test portion left on the neck of the flask. Gently mix the contents of the Kjeldahl flask.

### 9.1.2 Determination

#### 9.1.2.1 Digestion

Turn on the fume extraction system of the digestion apparatus (6.10) prior to beginning the digestion. Heat the Kjeldahl flask and its contents (9.1.1) on the digestion apparatus using a heater setting low enough such that charred digest does not foam up the neck of the Kjeldahl flask. Digest at this heat setting until white fumes appear in the flask after approximately 20 min. Increase the heater setting to half way to the maximum setting determined in 6.10 and continue the heating period for 15 min. At the end of the 15 min period, increase the heat to maximum setting determined in 6.10. After the digest clears (clear with light blue-green colour) continue boiling for 1 h to 1,5 h at maximum setting. If the liquid does not boil, the final burner setting may be too low. The total digestion time will be between 1,8 h and 2,25 h. If any charred digest is still left on the neck, rinse it with a few millilitres of water.

To determine the specific boiling time required for analysis conditions in a particular laboratory using a particular set of apparatus, select a high-protein, high-fat milk sample and determine its protein content using different boil times (1 h to 1,5 h) after clearing. The mean protein result increases with increasing boil time, becomes consistent and then decreases when boil time is too long. Select the boil time that yields the maximum protein result.

At the end of digestion, the digest shall be clear and free of undigested material. Allow the digest to cool to room temperature in an open flask under a separate hood over a period of approximately 25 min. If the flask is left on the hot burners to cool, it will take longer to reach room temperature. The cooled digest should be liquid or liquid with a few small crystals at the bottom of the flask at the end of 25 min cooling period. Excessive crystallization after 25 min is the result of undue acid loss during digestion and can result in low test values.

NOTE Undue acid loss is caused by excessive fume aspiration or an excessively long digestion time caused by digestions for too long a period at temperatures below the maximum temperature of the analysis.

To reduce acid loss, reduce the rate of fume aspiration. Do not leave the undiluted digest in the tubes overnight. The undiluted digest may crystallize during this period and it will be very difficult to get the crystallized digest back into solution.

Add 300 ml of water to the 500 ml Kjeldahl flasks or 400 ml of water when using the 800 ml Kjeldahl flasks. Use the water to also wash down the neck of the flask. Mix the contents thoroughly ensuring that any crystals that separate out are dissolved. Add 5 to 10 boiling aids (6.9). Allow the mixture to cool again to room temperature prior to the distillation. Diluted digests may be stoppered and held for distillation at a later time.

#### 9.1.2.2 Distillation

Turn on the condenser water for the distillation apparatus (6.11). Add 75 ml of sodium hydroxide solution (5.4) to the diluted digest (9.1.2.1) by carefully pouring the solution down the inclined neck of the Kjeldahl flask to form a layer at the bottom of the bulb of the flask. There should be a clean interface between the two solutions.

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To reduce the possibility of ammonia loss, immediately, after the addition of the sodium hydroxide solution to the Kjeldahl flask, connect it quickly to the distillation apparatus (6.11). Immerse the tip of the condenser outlet tube in 50 ml of the boric acid solution (5.6) contained in a conical flask (6.5).

Vigorously swirl the Kjeldahl flask to mix its contents thoroughly until separate layers of solution are no longer visible in the flask. Set the flask down on the burner.

Turn the burner of the steam generator up to a setting high enough to boil the contents of the Kjeldahl flask. Continue distillation until irregular boiling (bumping) starts and then immediately disconnect the Kjeldahl flask and turn off the burner. Turn off the condenser water. Rinse the in- and outside of the tip of the outlet tube with water collecting the rinsings in the conical flask and mix.

The distillation rate shall be such that approximately 150 ml of distillate is collected before irregular boiling (bumping) starts. The total volume of the contents of the conical flask will be approximately 200 ml. If the volume of distillate collected is less than 150 ml, then it is likely that less than 300 ml of water was added to dilute the digest. The efficiency of the condenser shall be such that the temperature of the contents of the conical flask does not exceed 35 °C during the distillation when using a colorimetric end-point.

#### 9.1.2.3 Titration

Using a burette (6.6), titrate the contents of the conical flask (9.1.2.2) against hydrochloric acid (5.7). The end-point is reached at the first trace of pink colour in the contents. Estimate the burette reading at least to the nearest 0,05 ml. An illuminated magnetic stirrer plate may aid visualization of the end-point.

Alternatively, titrate the contents of the conical flask (9.1.2.2) against hydrochloric acid (5.7) using a proper calibrated automatic titrator provided with a pH meter (6.17). The pH end-point of the titration is reached at pH 4,6, being the steepest point in the titration curve (inflection point). Read the amount of the used titrant on the automatic titrator.

NOTE 1 The first trace of pink is observed between pH 4,6 and pH 4,3 for the indicator system (5.5) and boric acid solution (5.6) specified in this method. In practice, the rate of change of pH as a function of hydrochloric acid (5.7) added is very fast within this pH range. It takes about 0,05 ml of 0,1 mol/l hydrochloric acid to change pH by 0,3 units in the range pH 4,6 to pH 4,3 in this system.

NOTE 2 The within and between laboratory method performance statistics for this method were determined using a colour end-point titration. Comparing the final test results, including those for their blank tests, obtained with a pH 4,6 end-point with those of a colour end-point titration showed that, statistically, no significant difference was demonstrable between them.

## 9.2 Block digestion method

### 9.2.1 Test portion and pre-treatment

Add to a clean and dry digestion tube (6.13), 12,0 g of potassium sulfate (5.1), 1,0 ml of copper(II) sulfate solution (5.2), the prepared test sample as indicated in Clause 8, and 20 ml of sulfuric acid (5.3) while using the sulfuric acid to also wash down any copper(II) sulfate solution, or potassium sulfate or test portion left on the upper walls of the digestion tube. Gently mix the contents of the tube.

Volumes of acid greater than 20 ml in the block digestion systems gave excessive foaming problems during digestion and variable results. Users of the block digestion method should note that maintaining sufficient residual sulfuric acid in the tube at the end of digestion needs more attention by the analyst than in traditional systems. Excessive acid loss due to overaspiration of fumes is more of concern in block digestors than traditional systems.

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### 9.2.2 Determination

#### 9.2.2.1 Digestion

Set the digestion block (6.12) at a low initial temperature to control foaming (between 180 °C and 230 °C). Transfer the tube to the digestion block and place the exhaust manifold (6.14) which itself is connected to a centrifugal scrubber of similar device (6.15), in the top of the tube. The aspiration rate of the centrifugal scrubber or similar device shall be just sufficient to remove fumes. The complete digestion apparatus may need to be kept inside a fume hood.

Digest the test portion for 30 min or until white fumes develop. Then increase the temperature of the digestion block to between 410 °C and 430 °C. Continue digestion of the test portion until the digest is clear.

It may be necessary to increase the temperature gradually over a period of approximately 20 min to control foaming. In any event, do not let foam rise higher than 40 mm to 50 mm below the surface of the exhaust manifold inserted into the top of the digestion tube.

After the digest clears (clear with light blue-green colour), continue digestion at between 410 °C and 430 °C for at least 1 h. During this time period the sulfuric acid shall boil. If visible boiling of the clear liquid is not apparent as bubbles forming at the surface of the hot liquid around the perimeter of the tube, then the temperature of the block may be too low. The total digestion time will be at between 1,75 h and 2,5 h.

To determine the specific boiling time required for analysis conditions in a particular laboratory using a particular set of apparatus, select a high-protein, high-fat milk sample and determine its protein content using different boil times (1 h to 1,5 h) after clearing. The mean protein result increases with increasing boil time, becomes consistent and then decreases when boil time is too long. Select the boil time that yields the maximum protein result.

At the end of digestion, the digest shall be clear and free of undigested material. Remove the tube from the block with the exhaust manifold in place.

Allow the digest to cool to room temperature over a period of approximately 25 min. The cooled digest should be liquid or liquid with a few small crystals at the bottom of the tube. Excessive crystallization after 25 min is the result of undue acid loss during digestion and can result in low test values.

NOTE Undue acid loss is caused by excessive fume aspiration or an excessively long digestion time caused by digestions for too long a period at temperature below the maximum temperature of the analysis.

To reduce acid loss, reduce the rate of fume aspiration. Do not leave the undiluted digest in the tubes overnight. The undiluted digest may crystallize during this period and it will be very difficult to get the crystallized digest back into solution.

After the digest has cooled to room temperature in approx. 25 min, remove the exhaust manifold and carefully add 85 ml of water to each tube. Swirl to mix while ensuring that any separated-out crystals are dissolved. Allow the contents of the tube to cool to room temperature again.

#### 9.2.2.2 Distillation

Turn on the condenser water for the distillation apparatus. Attach the digestion tube containing the diluted digest to the distillation unit (6.16). Place a conical flask (6.5) containing 50 ml of boric acid solution (5.6) under the outlet of the condenser, in such a way that the outlet is below the surface of the boric acid solution. Adjust the distillation unit to dispense 55 ml of sodium hydroxide solution (5.4).

In cases where a 40 % mass fraction sodium hydroxide solution (5.4) is used, adjust the dispensed volume to 65 ml. If the automatic delivery of sodium hydroxide solution is extremely variable due to partial plugging of the delivery tubing for the sodium hydroxide, then large variability between individual results occurs.

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Having due regard to the manufacturer's instructions, operate the distillation unit in such a way as to steam distil the ammonia liberated by addition of the sodium hydroxide solution, collecting the distillate in the boric acid solution. Continue with the distillation process until at least 150 ml of distillate is collected.

Remove the conical flask from the distillation unit and completely drain the distillation tip. Rinse the in- and outside of the tip with water collecting the rinsing in the conical flask. Rinse the tip always with water between samples. The efficiency of the condenser shall be such that the temperature of the contents of the conical flask does not exceed 35 °C during the distillation when using a colorimetric end-point.

#### 9.2.2.3 Titration

Using a burette (6.6), titrate the contents of the conical flask (9.2.2.2) against hydrochloric acid (5.7). The end-point is reached at the first trace of pink colour in the contents. Estimate the burette reading at least to its nearest 0,05 ml. An illuminated magnetic stirrer plate may aid visualization of the end-point.

Alternatively, using a properly calibrated automatic titrator provided with a pH meter (6.17), titrate the contents of the conical flask (9.2.2.2) against hydrochloric acid (5.7). The pH end-point of the titration is reached at pH 4,6, being the steepest point in the titration curve (inflection point). Read the amount of the used titrant on the automatic titrator.

NOTE 1 The first trace of pink is observed between pH 4,6 and pH 4,3 for the indicator (5.5) and boric acid solution (5.6) specified in this method. In practice, the rate of change of pH as a function of hydrochloric acid (5.7) added is very fast within this pH range. It takes about 0,05 ml of 0,1 mol/l hydrochloric acid to change pH by 0,3 units in the range pH 4,6 to pH 4,3 in this system.

NOTE 2 The within and between laboratory method performance statistics for this method were determined using a colour end-point titration. Comparing the final test results, including those for their blank tests, obtained with a pH 4,6 end-point with those of a colour end-point titration showed that, statistically, no significant difference was demonstrable between them.

### 9.3 Blank test

Always titrate blanks against the same hydrochloric acid (5.7) and using the same burette (6.6) or automatic titrator provided with a pH meter (6.17) as used for the test portions. Carry out a blank test according to 9.1 or 9.2. Replace the test portion with 5 ml of water and about 0,85 g of sucrose (5.10).

Keep a record of blank values. If blank values change, identify the cause.

NOTE 1 The purpose of the sucrose in a blank or a recovery standard is to act as organic material to consume an amount of sulfuric acid during digestion that is roughly equivalent to a test portion. If the amount of residual free sulfuric acid at the end of digestion is too low, the recovery of nitrogen by both recovery tests in 9.4.2 and 9.4.3 will be low. If, however, the amount of residual acid present at the end of digestion is sufficient to retain all the nitrogen, but the temperature and time conditions during digestion were not sufficient to release all the nitrogen from a sample, the nitrogen recovery in 9.4.2 will be acceptable and the nitrogen recovery in 9.4.3 will be low.

NOTE 2 The amount of titrant used in the blank should always be greater than 0,00 ml. Blanks within the same laboratory should be consistent across time. If the blank is already pink before the beginning of titration, something is wrong. Usually in such cases, the conical flasks are not clean or water from the humid air that may condense on the outside of the condenser apparatus has dripped down into the collection flask to cause the contamination. Typical blank values are less than or equal to 0,2 ml.

### 9.4 Recovery tests

**9.4.1** Regularly check the accuracy of the procedure by means of the following recovery tests, carried out in accordance with 9.1 and 9.2.

**9.4.2** Check that no loss of nitrogen occurs by using a test portion of 0,12 g of ammonium sulfate (5.8) along with 0,85 g of sucrose (5.10).

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NOTE The ammonium sulfate recovery check does not give information about the capability of the digestion conditions to release nitrogen that is bound in the protein structures.

The percentage of nitrogen recovered shall be greater than 99 % mass fraction for all positions on the apparatus. For recoveries less than 99 %, the normality of the titrant is higher than the stated value, or nitrogen loss may have occurred in the digestion or distillation.

It is possible to use a mixture of ammonium sulfate and a small amount of sulfuric acid (the amount of residual remaining at the end of a digestion) in a Kjeldahl flask. Dilute it with the normal volume of water, add the normal amount of sodium hydroxide and distill. If the nitrogen recovery is still low by the same amount, the loss of nitrogen is in the distillation apparatus and not in that of the digestion. Probable causes are leaky tubing in a traditional system or failure to immerse the tips of the condensers in the boric acid solution early in the distillation. The apparatus shall pass this test positively before going on to check recoveries by the procedure in 9.4.3.

If recoveries of nitrogen exceed 100 % and no loss of nitrogen can be identified, some possible causes are:

a) the ammonium sulfate is contaminated;

b) the actual normality of the titrant is lower than its stated value;

c) the calibration of the burette for the titrant is wrong;

d) the temperature of the titrant is too far above the temperature of burette calibration; or

e) the flow of titrant out of the burette exceeds the maximum speed at which the burette calibration is valid.

NOTE Although the maximum theoretical recovery cannot exceed 100 %, in practice recoveries higher than the maximum may be obtained due to the uncertainty of measurements.

**9.4.3** Check the efficiency of the digestion procedure by using 0,16 g of lysine hydrochloride or 0,18 g of tryptophan (5.9) along with 0,67 g of sucrose (5.10). At least a mass fraction of 98 % of the nitrogen shall be recovered.

If the recovery is lower than 98 %, after having a mass fraction of 99 % to 100 % recovery on ammonium sulfate, then the temperature or time of digestion is insufficient or there is undigested sample material (i.e. char) on the inside of the Kjeldahl flask.

The final evaluation of performance is best done by participation in a proficiency-testing programme, where within and between laboratories statistical parameters are computed based on analysis of milk test samples.

**9.4.4** Lower results in either of the recovery tests (or higher than 100,0 % in 9.4.2) will indicate failures in the procedure and/or inaccurate concentration of the hydrochloric acid solution (5.7).

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## 10 Calculation and expression of results

### 10.1 Calculation

#### 10.1.1 Nitrogen content

Calculate the nitrogen content, $w_N$, expressed as a percentage by mass to four decimal places, of the test sample using Equation (1):

$$w_N = \frac{1{,}400\,7\left(V_s - V_b\right)c_t}{m} \quad (1)$$

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where

$c_t$ is the amount of substance concentration, in moles per litre, expressed to four decimal places, of the hydrochloric acid solution (5.7);

$V_b$ is the volume, in millilitres, to at least the nearest 0,05 ml, of the hydrochloric acid solution (5.7) used in the blank test (9.3);

$m$ is the mass, in grams, expressed to the nearest milligram, of the test portion (9.1);

$V_s$ is the volume, in millilitres, to at least the nearest 0,05 ml, of the hydrochloric acid solution (5.7) used in the determination (9.2.2.3).

#### 10.1.2 Crude protein content

Calculate the crude protein content, $w_p$, expressed as a percentage by mass, using Equation (2):

$$w_p = 6{,}38 w_N \quad (2)$$

where 6,38 is the generally accepted factor to convert nitrogen content to crude protein content.

### 10.2 Expression of results

Do not round test results further until the final use of the test value is made.

**IMPORTANT — This is particularly true when the values are going to be used for further calculation. One example is when the individual test values obtained from the analysis of many sample materials are used to calculate method performance statistics for within and between laboratory variation. Another example is when the values are used as a reference for instrument calibration (e.g. infrared milk analyser) where the values from many samples will be used in a simple or multiple regression calculation. In such cases, the obtained results should not be rounded before they are used for further calculations.**

#### 10.2.1 Nitrogen content

Express the test results to four decimal places, if needed for further calculations.

Otherwise, express the final test results to three decimal places.

#### 10.2.2 Crude protein content

Express the test results to three decimal places, if needed for further calculations.

Otherwise, express the final test results to two decimal places.

## 11 Precision

### 11.1 Interlaboratory test

An interlaboratory trial was conducted in accordance with ISO 5725-1[2] and ISO 5725-2[3]. The values for repeatability and reproducibility for a variety of cheeses, including reprocessed cheeses have been published (see Reference [5]).

A summary of results for processed cheeses included in the trial can be found in Annex A.

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